



**University of
Zurich^{UZH}**

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2013

Understanding the role of argininosuccinate lyase transcript variants in the clinical and biochemical variability of the urea cycle disorder argininosuccinic aciduria

Hu, Liyan ; Pandey, Amit V ; Eggimann, Sandra ; Rüfenacht, Véronique ; Möslinger, Dorothea ; Nuoffer, Jean-Marc ; Häberle, Johannes

Abstract: Argininosuccinic aciduria (ASA) is an autosomal recessive urea cycle disorder caused by deficiency of argininosuccinate lyase (ASL) with a wide clinical spectrum from asymptomatic to severe hyperammonemic neonatal onset life-threatening courses. We investigated the role of ASL transcript variants in the clinical and biochemical variability of ASA. Recombinant proteins for ASL wild type, mutant p.E189G, and the frequently occurring transcript variants with exon 2 or 7 deletions were (co-)expressed in human embryonic kidney 293T cells. We found that exon 2-deleted ASL forms a stable truncated protein with no relevant activity but a dose-dependent dominant negative effect on enzymatic activity after co-expression with wild type or mutant ASL, whereas exon 7-deleted ASL is unstable but seems to have, nevertheless, a dominant negative effect on mutant ASL. These findings were supported by structural modeling predictions for ASL heterotetramer/homotetramer formation. Illustrating the physiological relevance, the predominant occurrence of exon 7-deleted ASL was found in two patients who were both heterozygous for the ASL mutant p.E189G. Our results suggest that ASL transcripts can contribute to the highly variable phenotype in ASA patients if expressed at high levels. Especially, the exon 2-deleted ASL variant may form a heterotetramer with wild type or mutant ASL, causing markedly reduced ASL activity.

DOI: <https://doi.org/10.1074/jbc.M113.503128>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-91950>

Journal Article

Accepted Version

Originally published at:

Hu, Liyan; Pandey, Amit V; Eggimann, Sandra; Rüfenacht, Véronique; Möslinger, Dorothea; Nuoffer, Jean-Marc; Häberle, Johannes (2013). Understanding the role of argininosuccinate lyase transcript variants in the clinical and biochemical variability of the urea cycle disorder argininosuccinic aciduria. *Journal of Biological Chemistry*, 288(48):34599-34611.

DOI: <https://doi.org/10.1074/jbc.M113.503128>

Molecular Bases of Disease:
Understanding the Role of
Argininosuccinate Lyase Transcript
Variants in the Clinical and Biochemical
Variability of the Urea Cycle Disorder
Argininosuccinic Aciduria

Liyan Hu, Amit V. Pandey, Sandra Eggimann,
Véronique Rüfenacht, Dorothea Möslinger,
Jean-Marc Nuoffer and Johannes Häberle
J. Biol. Chem. published online October 17, 2013



Access the most updated version of this article at doi: [10.1074/jbc.M113.503128](https://doi.org/10.1074/jbc.M113.503128)

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
<http://www.jbc.org/content/early/2013/10/17/jbc.M113.503128.full.html#ref-list-1>

Understanding the Role of Argininosuccinate Lyase Transcript Variants in the Clinical and Biochemical Variability of the Urea Cycle Disorder Argininosuccinic Aciduria

Liyan Hu^{1,2}, Amit V Pandey³, Sandra Eggimann^{4,5}, Véronique Rüfenacht^{1,2}, Dorothea Möslinger⁶, Jean-Marc Nuoffer^{4,5}, and Johannes Häberle^{1,2}

¹Division of Metabolism, University Children's Hospital, 8032 Zurich, Switzerland

²Children's Research Center, 8032 Zurich, Switzerland

³Pediatric Endocrinology, Department of Clinical Research, University of Bern, 3010 Bern, Switzerland

⁴University Institute of Clinical Chemistry, University of Bern, 3010 Bern, Switzerland

⁵University Children's Hospital, University of Bern, 3010 Bern, Switzerland

⁶Department of Paediatrics and Adolescent Medicine, Medical University of Vienna, 1090 Vienna, Austria

*Running title: *Role of ASL Transcript Variants in ASA*

Corresponding author: Johannes Häberle, University Children's Hospital Zurich, Division of Metabolism, Steinwiesstrasse 75, 8032 Zurich, Switzerland, Tel.: +41 44 266 7342; Fax: +41 44 266 7167; Email: Johannes.Haeberle@kispi.uzh.ch

Key words: Computer modeling; Metabolic diseases; Recombinant protein expression; Transfection; Urea Cycle; Argininosuccinic Aciduria (ASA); Argininosuccinate Lyase (ASL); Homo-/Heterotetrameric protein; Transcript variants

Background: The role of argininosuccinate lyase (ASL) transcripts in disease variability is unclear.

Results: The most common ASL transcript variants decrease the functional enzymatic activity after co-expression with wildtype or mutant ASL.

Conclusion: ASL transcripts expressed at high levels can contribute to the variable phenotype in ASL-deficient patients.

Significance: A new explanation of molecular basis adds to our understanding of the clinical variability in patients.

Abstract

Argininosuccinic aciduria (ASA) is an autosomal recessive urea cycle disorder caused by deficiency of argininosuccinate lyase (ASL) with a wide clinical spectrum from asymptomatic to severe hyperammonemic neonatal onset life-threatening courses. We investigated the role of ASL transcript variants in the clinical and biochemical variability of ASA. Recombinant proteins for ASL wildtype, mutant p.Glu189Gly and the frequently occurring transcript variants with exon 2 or 7 deletions were (co)-expressed in human embryonic kidney 293T cells. We found that exon 2-

deleted ASL forms a stable truncated protein with no relevant activity but a dose-dependent dominant negative effect on enzymatic activity after co-expression with wildtype or mutant ASL, while exon 7-deleted ASL is unstable but seems to have, nevertheless, a dominant negative effect on mutant ASL. These findings were supported by structural modelling predictions for ASL hetero/homotetramer formation. Illustrating the physiological relevance, the predominant occurrence of exon 7-deleted ASL was found in two patients who were both heterozygous for the ASL mutant p.Glu189Gly. Our results suggest that ASL transcripts can contribute to the highly variable phenotype in ASA patients if expressed at high levels. Especially, the exon 2-deleted ASL variant may form a heterotetramer with wildtype or mutant ASL causing markedly reduced ASL activity.

Introduction

Argininosuccinate lyase (ASL; EC 4.3.2.1; OMIM *608310) catalyzes the reversible hydrolytic cleavage of argininosuccinate into arginine and fumarate and contributes to removal of waste nitrogen and biosynthesis of arginine within the urea cycle in ureotelic species (1). ASL is also involved in the arginine-

citrulline cycle as part of a multi-protein complex required for production of nitric oxide (2) as well as in other pathways (Fig. 1).

The human *ASL* gene is located on chromosome 7q11.21 (3-4) and comprises 16 exons encoding 464 amino acids (5-6). The resulting monomers have a predicted molecular mass of ~52 kDa and form a homotetrameric functional enzyme with four active sites (7). ASL has significant homology to δ -crystallin with an amino acid sequence identity of 64-71% between human ASL and various δ -crystallins (8-9). The δ -crystallins are major structural components of avian and reptilian eye lenses and show significant ASL enzyme activity in duck and chicken (9-10). Human ASL is expressed predominantly in liver (11) but is also detected in many other tissues including kidney (12), small intestine (13-14), pancreas and muscle (15), heart (16), brain (17-18), skin fibroblasts (19) and erythrocytes (20).

Mutations in the *ASL* gene result in an autosomal recessive disorder known as argininosuccinic aciduria (ASA; synonymous ASL deficiency, ASLD; OMIM #207900) (21) which is the second most common disorder in the urea cycle with an estimated incidence of approximately 1 per 70,000 live births (22). The clinical and biochemical phenotype of ASA is highly variable ranging from asymptomatic cases with only a biochemical phenotype (23-25), some of them diagnosed through newborn screening, to severe neonatal-onset hyperammonemic encephalopathy (26-27). The molecular basis for the diversity of ASA is not fully understood and several explanations have been suggested including tissue specific ASL expression (27-28), genetic heterogeneity at the *ASL* locus (29), intragenic complementation (7,30-32), different levels of residual ASL activity (33-34), the developmental control of the *ASL* gene by DNA methylation (35), and alternative splicing events at the *ASL* locus leading to frequent exon deletions (5,36-37).

In this study we explored the role of naturally occurring ASL transcript variants in the formation and function of ASL homotetramer to better understand the phenotypic variability of ASA. By combining computational structural analysis using molecular dynamic simulations and eukaryotic (co-)expression of wildtype (wt) with the most common transcript variants formed by deletions of exons 2 or 7, we could show that exon 2-deleted (ex2del) or exon 7-deleted (ex7del) ASL has a dominant negative effect on the ASL activity after co-expression

with wildtype or mutant ASL, respectively. Suggesting a physiological role of transcript variants, RNA analysis revealed a predominant expression of ex7del ASL in two ASA patients identified with heterozygous for the ASL mutant p.Glu189Gly. Taken together, these findings suggest that the frequent occurrence of ASL transcript variants, when they are expressed at high levels, can be a factor contributing to the highly variable clinical and biochemical phenotype of ASA. In particular, the effect may be even more striking in the stable mutant such as ex2del ASL variant since it may form a heterotetramer with ASL wildtype or naturally occurring missense mutations (i.e. sequence alterations with a disease-causing role found in ASL deficient patients) contributing to reduced ASL activity.

Experimental Procedures

ASL transcript expression in different tissues

A panel of cDNAs from 17 different human tissues comprising ASL patient fibroblasts and 16 other tissues (Multiple Tissue cDNA Panel Human I and II, Clontech, Mountain View CA, USA) was used for the amplification of full-length ASL as well as shorter RNA fragments. In addition, short ASL cDNA fragments derived from skin fibroblasts of 24 ASA patients with 10 different genotypes were amplified. Specific oligonucleotides

ASL-FL-F-5'acgaggaaccgcccaacat3' (forward) and ASL-FL-R-5'tgcctctccagtcctgactgt3' (reverse) were used for amplification of full-length ASL; primers ASL-SF-F-5'acataatccgtgcggccagg3' (forward, derived from ASL 5'-UTR-sequence) and ASL-SF-R-5'tgagatgggtcatgcacagcg3' (reverse, derived from ASL exon 10 sequence) were used for amplification of a short fragment containing exons 1-9 with a fragment size of 864 bp. Hot start PCR was performed at an annealing temperature of 60°C (62°C for primers ASL-SF-F with ASL-SF-R) for 38 cycles (42 cycles for primers ASL-SF-F with ASL-SF-R) using HOT FIREPol® DNA polymerase (Solis Biodyne, Tartu, Estonia) or HotStarTaq DNA Polymerase (QIAGEN GmbH, Hilden, Germany) prior to gel electrophoresis. PCR products representing probable splice variants were confirmed by sequencing using the BigDye Terminator cycle sequencing kit V.1.1 (Applied Biosystems, ABI sequence).

Patients or their legal guardians had consented to the use of the cultured skin fibroblasts for

research purposes when the skin biopsy was taken and cDNA samples were anonymized prior to their use in this study.

Patients' characteristics and RNA studies

The 19-year-old male patient, offspring of non-consanguineous parents, was first identified at the age of 13 years with mild hyperammonemia (250 $\mu\text{mol/L}$, normal <50), confusion and irritability during an episode of gastroenteritis. The diagnosis of ASA was made based on characteristic urine metabolites and underlined by typical signs and symptoms such as mild hepatomegaly but normal liver function, trichorrhexis nodosa, cognitive impairment (IQ 67), and a natural avoidance of protein rich food since early childhood. Besides, there was persistent mild elevation of plasma citrulline (between 80 and 150 $\mu\text{mol/L}$, normal <60). Under treatment with L-arginine (150 mg/kg/day) and mild protein restriction he was stable ever since apart from a single mild metabolic decompensation at age 17 years again during gastroenteritis with maximum ammonia (230 $\mu\text{mol/L}$).

To confirm the diagnosis, DNA sequencing of the *ASL* gene by standard methods (6) as well as array comparative genomic hybridization (38) for exclusion of a deletion on the second allele revealed only a single heterozygous mutation in exon 7 (c.566A>G, p.Glu189Gly) which was not present on the maternal allele (also, no other mutation was found in the mother in DNA or RNA) while the mutation was found in a heterozygous state in paternal DNA. To identify the second mutant allele, RT-PCR was performed using RNA (PrimeScript II 1st Strand cDNA Synthesis kit, TaKaRa Bio) derived from a 3-days full blood culture treated with phytohemagglutinin and cycloheximide (39). For control-RT-PCR, cDNAs derived from lymphocytes, liver and fibroblasts were used. The patient's cDNA was amplified in *ASL* full length as well as in a short fragment comprising exons 1 to 9 as described above. In addition, primers *ASL*-SF-F and *ASL*-SF-R7-5'ctcccaggggcaggacattg3' (reverse, derived from *ASL* exon 7 sequence) were used for amplification of a short fragment containing exons 1-7 (fragment size of 670 bp) to confirm mutation c.566A>G in this transcript. PCR products were sequenced as described above.

In another patient (aged 12 years) with late onset ASA and a similar clinical and biochemical situation, the mutation p.Glu189Gly was found

in a heterozygous state and the same DNA and RNA investigations were performed as above.

To estimate expression levels of transcripts, densitometry analysis of RT-PCR products on gel electrophoresis and of bands detected by Western blotting was performed by using Carestream Molecular Imaging software (Carestream Health, Germany).

The genetic studies were done after written informed consent of the patient and his legal guardians was obtained.

Generation of wildtype, mutant ASL p.Glu189Gly and exon 2 or 7 deleted splice variant constructs

Full-length *ASL* cDNA (1395 bp) and ex7del *ASL* transcript variant (1317 bp) were cut from pCR2.1 carrying the wt *ASL* (33) and pCMV6-XL4 carrying the ex7del *ASL* cDNA (OriGene, Rockville, MD, USA) using restriction enzymes *Bam*HI and *Not*I (both New England Biolabs, Beverly, MA, USA), respectively. Restriction products were cloned into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) yielding pcDNA3-*ASL*-wt (P-wt) and pcDNA3-*ASL*-ex7del (P-ex7del), respectively. Oligonucleotides *ASL*-ex2del-F-5'atccggtccatggcctcggaggtggctgaggagtgggcc3' (forward, consisting of a *Bam*HI site, the 12 nucleotides (nt) of *ASL* exon 1 and 18 nt from 5' exon 3) and *ASL*-ex2del-R-5'cctctagatgcattctcgcgcggccgctatatctaggc3' (reverse, with a *Not*I site added to an *ASL* 3'-UTR-sequence) were used to amplify the exon2-deleted *ASL* cDNA from P-wt. The obtained PCR product was gel-purified and cloned into the above expression vector yielding pcDNA3-*ASL*-ex2del (P-ex2del). The mutant p.Glu189Gly (c.566A>G) was constructed as pcDNA3-*ASL*-Glu189Gly (P-E189G) based on P-wt by site-directed mutagenesis (Phusion Site-directed mutagenesis Kit, Finnzymes, Espoo, Finland) according to manufacturer's protocol. All established constructs were confirmed by sequencing as above.

Expression and co-expression of ASL constructs in human embryonic kidney 293T cells

Three different mammalian cell lines (COS-1, HeLa and 293T) were tested for their background *ASL* activity before establishing the eukaryotic *ASL* expression system. *ASL* expression levels after transfection with P-wt were determined by Western blot. Cells were grown in Dulbecco's modified Eagle's medium + GlutaMAX (DMEM, Gibco, Paisley, UK)

supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (both PAA, Pasching, Austria) and maintained in an incubator containing 5% CO₂ at 37°C in a humidified atmosphere.

Based on the lowest ASL background activity, 293T cells were considered as ideal for the ASL expression system in this study. The cells were transiently (48 hours) transfected with total 7 µg of the constructs P-wt, P-ex2del or P-ex7del in 60 mm dishes using Lipofectamine™ LTX and PLUS™ reagents according to the manufacturer's instructions (Invitrogen, Basel, Switzerland). Total 7 µg or 10.5 µg of plasmids were used for co-transfection with 2 or 3 plasmids; if not indicated otherwise, we used the same amount of respective constructs (each 3.5 µg) in co-transfectants. For the co-transfection of P-wt and P-ex2del at different ratios, 1.75 µg P-wt and 1.75 µg P-ex2del was used for the ratio 1:1; 1.75 µg P-wt and 3.5 µg P-ex2del for 1:2; 1.75 µg P-wt and 8.75 µg P-ex2del for 1:5; 3.5 µg P-wt and 1.75 µg P-ex2del for 2:1; 8.75 µg P-wt and 1.75 µg P-ex2del for 5:1. The empty vector (EV) pcDNA3 was used either as negative control or to set up same amounts of total plasmids for co-transfection.

RNA was isolated from the cells transiently transfected with P-wt, P-ex2del or P-ex7del, respectively, using QIAamp RNA blood minikit according to the manufacturer's protocol (QIAGEN GmbH, Hilden, Germany). The concentration of nucleic acids was determined by Nanodrop. RT-PCR was performed as standard protocol using primers ASL-SF-F3-5'atccacacagccaatgagcgc3' (forward, derived from ASL exon 3 sequence) and ASL-SF-R for amplification of a short fragment containing exons 4-9 with a fragment size of 535 bp.

Protein extraction, Western blot analysis and Immunoprecipitation

Cells were harvested and lysed in Lubrol WX lysis buffer containing 0.15% (w/v) of Lubrol WX (Sigma Chemical Co., Poole, Dorset, UK) and 10 mM of Tris-HCl (pH 8.6). Human liver tissue (n=3, shock frozen needle biopsy samples for diagnostic purposes in non ASLD patients after informed consent into scientific use was obtained) was homogenized in complete Nonidet P40 (NP-40, Roche Diagnostics GmbH, Mannheim, Germany) lysis buffer containing 1% of NP-40, 50 mM of Tris-HCl (pH 8), 125 mM of NaCl, 1 mM of Ethylenediaminetetraacetic acid (EDTA) and protease inhibitors (1x Complete EDTA-free + 1

mM of phenylmethyl-sulfonyl fluoride, PMSF) (Roche Diagnostics GmbH, Mannheim, Germany) in a pre-chilled glas-grinder by quickly grinding on ice. Cell lysates and liver homogenates were then centrifuged at maximum speed at 4°C for 15 min. Protein concentrations in the supernatants (= cell extracts) were determined by the method of Lowry (40) using bovine serum albumin (BSA) as a standard.

Western blotting was performed as previously described (41). 30 µg total protein of cell extracts was separated by 10% denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE (without SDS in the Laemmli loading buffer and electrophoresis buffer), and subsequently transferred to nitrocellulose transfer membranes (Whatman GmbH, Dassel, Germany). The primary polyclonal antibody anti-ASL (GeneTex, Irvine CA, USA), recognizing ASL residues 13 to 261 according to the manufacturer, was used at a dilution of 1:1000 and the horseradish peroxidase (HRP)-conjugated secondary antibody anti-rabbit (Santa Cruz Biotechnology, Santa Cruz CA, USA) was used at a dilution of 1:5000. Antibodies against β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (both Santa Cruz Biotechnology) served as loading controls. Protein detection was done using ECL reagents (GE Healthcare, Glattbrugg, Switzerland) for chemiluminescent labelling.

Fibroblasts derived from 3 ASA patients as well as one control were cultured under the same condition as for 293T cells. Immunoprecipitation analysis was done as standard protocol using 5 mg of total protein of fibroblasts followed by Western blot analysis as described above. HRP-conjugated Mouse Anti-rabbit IgG (L27A9, Cell Signaling Technology, Inc. Danvers, USA), which does not recognize the denatured and reduced rabbit IgG heavy or light chains on Western blot, was used as secondary antibody at a working dilution of 1:2000.

Measurement of ASL enzymatic activity

The ASL enzyme activity was determined spectrophotometrically in cell extracts after transient transfection or co-transfection of P-wt, P-ex2del and/or P-ex7del, using a coupled assay with arginase and measuring urea production as described before (33). Briefly, 100 µl of 34 mM argininosuccinate (argininosuccinic acid disodium salt hydrate) in water and 100 µl arginase (50 units) (both Sigma-Aldrich, Buchs, Switzerland) in 66.7 mM phosphate buffer (11.1

mM potassium dihydrogenphosphate and 55.6 mM disodium hydrogenphosphate, pH 7.5) were incubated at 37°C for 5 min. Then 40 µl cell extract (3-14 µg total protein) and 10 µl phosphate buffer were incubated with the above reagents at 37°C for 30 min. The reaction was stopped by adding perchloric acid at a final concentration of 2%. The ASL enzyme activities are given as mIE/mg total protein. The residual ASL activities of splicing variants are determined as percentage of ASL-wt or ASL-wt with EV activity (%) in each (co-)transfection under same condition, respectively. All assays were carried out in triplicate for at least 3 independent co-transfection experiments.

Structure modelling

3D protein model and in-silico mutagenesis of ASL – The tetrameric 3D structural model of ASL (NCBI NP_000039.2, Uniprot P04424) sequence (AA 1-464) was built using the ASL structure (1K62) as template. Model building was performed with programs YASARA (42) and WHATIF (43). Side chains in newly built parts were optimized by molecular dynamic (MD) simulations. The geometry information for the tetramer was extracted from the original crystallographic data. The final model was refined by a 1000 ps (MD) simulation using AMBER 2003 force field and checked with the programs WHAT_CHECK (44), WHATIF (43), Verify3D (45-46), and Ramachandran plot analysis (47-48). To create the exon 2 and exon 7 deletions we first made the FASTA sequence files of modified proteins and performed model building using both the monomer and tetramer structures and performed refinements as described for wt protein. Structures were depicted with Pymol (www.pymol.org). Structural properties of the proteins were calculated by YASARA and WHATIF and general protein parameters were calculated with Expasy protein tools (www.expasy.ch)

Molecular Dynamics simulation for model refinement - The MD simulations were performed using AMBER03 force field (42). The simulation cell was filled with water, pH was fixed to 7.4 and the AMBER03 (49) electrostatic potentials were evaluated for water molecules in the simulation cell and adjusted by addition of sodium and chloride ions. The final MD simulations were then run with AMBER03 force field at 298K, 0.9% NaCl and pH 7.4 for 1000 ps to refine the models. Best models were selected for analysis and evaluation of the effect

of exon deletions on monomer and tetramer structures.

Statistics

Statistical analyses were done using percentages of ASL-wt activities by One-way ANOVA with the program GraphPad Prism 4 (GraphPad Software, San Diego, CA) to describe the differences of ASL activities between cells co-expressing ASL-wt with EV and cells co-expressing ASL-wt with the transcript variants. Differences were considered as significant if p value was < 0.05.

Results

Expression of wildtype ASL and of transcript variants in different tissues

To investigate whether the expression of ASL transcript variants is tissue-dependent and to confirm the reported occurrence of transcripts with deletions of exon 2 or 7 (5,36-37), we amplified ASL cDNA either in full-length (Data not shown) or in short cDNA fragments comprising exons 1-9 from 17 different human tissues (only 16 tissues shown in Fig. 2A) and a series of 24 skin fibroblast cell lines from ASA patients representing 10 different genotypes (Fig. 2B). In all tissues, wt ASL cDNA (short fragment with 864 bp) and, in addition in most tissues shorter transcript variants (786 bp, 669 bp) could be detected (Fig. 2A,B). ASL-wt cDNA was predominantly found in liver (Fig. 2A: lane 5) and kidney (Fig. 2A: lane 7), but expression was detected in all tissues. Furthermore, a similar expression pattern of ASL transcript variants was detected at low level in all cDNAs investigated for short fragments (Fig. 2A,B). Sequencing analysis of the shorter fragments identified them as exon 2- (669 bp) or exon 7-deleted (786 bp) transcripts.

RNA studies reveal predominant expression of exon 7-deleted ASL transcript variant in ASA patients

In RNA from both patients, ASL-ex7del variant (786 bp) was predominantly expressed (Fig. 2C: lane 2, 3; Fig. 2D: lane 16). Estimation of expression levels by densitometry yielded similar levels of the mutant variant (114% for Fig. 2C: lane 2 and 83% for Fig. 2C: lane 3) when compared to wt ASL (864 bp) in lymphocyte control (Fig. 2C: lane 4, set to 100%) but much higher levels (330% for Fig. 2C: lane 2 and 182% for Fig. 2C: lane 3) if

compared with the full-length band in the same patients (Fig. 2C: lane 2 and 3, set to 100%). Notably, the full-length band was expressed much less (34% for Fig. 2C: lane 2 and 46% for Fig. 2C: lane 3) compared to wt in lymphocyte control (Fig. 2C: lane 4, set to 100%). Expression levels of transcript variants in controls (Fig. 2C: lane 4; Fig. 2D: lane 1-15) and also in the patient's mother (Fig. 2C: lane 1; Fig. 2D: lane 17) were much lower in all tissues compared to wt (lymphocytes 9%, liver 6%, fibroblasts 10% as shown in Fig. 2D lanes 1-5, 6-10, 11-15, respectively). Sequencing confirmed that the variants represented ex2del or ex7del transcripts. To facilitate further sequencing, only a small fragment comprising exons 1-7 was amplified using a reverse primer derived from exon 7. Hereby, the mutation c.566A>G was confirmed in a hemizygous state in one patient (Fig. 2C: lane 2; Fig. 2E) and in a heterozygous state in the other patient (Fig. 2C: lane 3; Fig. 2F).

Endogenous ASL expression in mammalian cell lines and in human liver

To establish an ASL expression system in mammalian cells lacking endogenous but allowing for high ectopic ASL expression, we transiently transfected P-wt as well as EV pcDNA3 into COS-1, 293T and HeLa cells, respectively. Western blot analysis indicated that 293T cells fulfilled the above criteria in an optimal way while COS-1 cells showed endogenous ASL expression and HeLa cells failed to express ASL after transfection (Fig. 3A). These results were further confirmed by analysis of ASL activity (ASL endogenous activity in 293T cells shown in Table 1). Although ASL splice variants (ASL-ex7del and ASL-ex2del) were present in all cDNAs from various tissues (Fig. 2A-D), there was no corresponding signal detected when human liver homogenates from controls were investigated by Western blot analysis (Fig. 3B). Likewise, immunoprecipitation did not yield a detectable wt signal when patients' and control fibroblasts were used (Data not shown). This points towards and is probably explained by the low level of ASL expression in this cell type which is obviously not suited for further investigation of ASL wildtype and mutants on the protein level.

Expression and co-expression of ASL wildtype, mutant and transcript variants in 293T cells

To study the role of naturally occurring ASL splice variants in wildtype as well as in mutant

p.Glu189Gly, we first introduced diverse ASL recombinant constructs P-wt, P-E189G, P-ex2del and P-ex7del into 293T cells to (co-) express ASL wt, mutant p.E189G, ex2del or ex7del ASL, respectively. At the protein level, expression of ASL-wt or ASL-ex2del was detected either as monomers (Fig. 3C: lane 3 and 4 in upper panel; Fig. 3D: lane 1 and 5 in upper panel, respectively) or as homotetramer (Fig. 3C: lane 3 and 4 in middle panel; Fig. 3E: lane 1 and 5 in upper panel, respectively). Moreover, ASL-ex2del could also be co-expressed with ASL-wt as well as with mutant p.E189G (Fig. 3D-G). Densitometry analysis of the detectable bands by Western blotting showed that ASL-wt was less expressed in cells co-expressed with transcript variants (71.7% in co-transfectant with ASL-ex2del in Fig. 3D: lane 2; 88.7% in co-transfectant with ASL-ex7del in Fig. 3D: lane 3; 71.2% in co-transfectant with ASL-ex2del and ASL-ex7del in Fig. 3D: lane 4; all done after normalisation according to expressed loading control GAPDH, respectively) compared to co-expression with EV (Fig. 3D: lane 1, set to 100%). Additionally, ASL-ex2del was expressed at higher levels (55.4%, 53.6%, 60% or 61%) than ASL-wt (44.6%, 46.4% or 40%) or mutant p.Glu189Gly (39%) (cells co-transfected with ASL-ex2del and ASL-wt in Fig. 3A: lane 2, 4; Fig. 3G: lane 4; cells co-transfected with ASL-ex2del and p.Glu189Gly in Fig. 3G: lane 6). However, ASL-ex7del was not found (Fig. 3C: lane 5; Fig. 3D,E: lane 6 in upper panel; Fig. 3G: lane 9, respectively) although ASL-ex7del-RNA derived from cells transfected with P-ex7del could be detected by RT-PCR (Data not shown). These results suggest that deletion of ASL exon 2 results in a truncated but stable ASL protein while deletion of ASL exon 7 probably leads to an unstable protein.

Structure of ASL monomers and homotetramer

To study the structural implications of exon 2 and 7 deletions we made computational structural models of exon deleted ASL variants based on known structures of ASL available from RCSB database. We first performed a PhiBlast search of PDB database with the modified amino acid sequences to create a custom position specific scoring matrix (PSSM) that was then used in further runs of PhiBlast searches to identify structurally similar sequences. Then a secondary structure prediction of the original wt sequence was used to perform the structure based alignment of the sequences (Fig. 4). Aligned sequences were loaded in the

programs YASARA and WHATIF for generating structural models. Some of the loops in exon deleted sequences were modelled separately by scanning a library of loop databases. For our model building we used the information available in PDB_REDO database (<http://swift.cmbi.ru.nl/gv/pdbredo/index.html>) which re-refines the old structures in PDB database using latest methods based on original structural data deposited in PDB, and corrects the errors in structures found in PDB database. This is achieved by employing current state of the art refinement methods and software to the older crystallographic data. For the ASL structure (PDB #1K62), the overall Ramachandran plot appearance improved from -2.514 to -0.803 for the optimized entry, and total number of bumps/structural clashes were reduced from 150 in the original entry to 54 in optimized structure, Chi-1/Chi-2 rotamer normality improved from -2.186 to -0.576, 1st generation packing quality improved from 0.148 to 0.466, backbone conformation improved from -0.369 to -0.168 and R-free value changed from 0.2290 to 0.1909 in the fully optimised version used by us.

The ASL structure is composed of residues 5-464 of ASL protein. The enzymatically active ASL tetramer is formed by four identical subunits of ASL comprising of mainly alpha helices and contains three structurally distinct domains. Domains 1 and 3 have similar topology and contain two helix-turn-helix motifs while domain two has nine helices out of which five helices form the core monomer structure in an up-down-up-down-up sequence (Figure 5A). Two sets of dimers come together in antiparallel manner to form the tetramer (Fig. 5A, 6A). The core of the homotetramer is composed of a four-helix bundle with each monomeric subunit contributing one helix to the central core, and the tetramer is held together by hydrophobic interactions between the four central helices as well as ionic interactions between arginines and glutamic acid residues on two distinct dimeric structures.

Role of exon 2 residues

The amino acid residues contributed by exon 2 (5-69) form the N-terminus of the ASL protein (Fig. 4 and 5A) and are needed for the enzymatic activity as the previously described R12Q mutation in exon 2 has been shown to result in loss of activity. Two of the helices in domain 2 of ASL monomeric structure are formed by residues from exon 2 and are missing in

exon2del variant of ASL (Fig. 5B). Most of the N-terminal chain in the ASL monomer is flexible and during molecular dynamic simulations it was found to fluctuate during the whole run indicating a dynamic arrangement in both the monomeric and tetrameric form. There were few contacts between the N-terminal loops of any individual subunit to other subunits in the tetrameric structure, indicating that it probably does not influence tetramer formation. The role of N-terminus residues on the catalytic activity is not clear but one side of the active site is covered by these residues (Fig. 6B,C). The flexible nature of amino acids 5-18 suggests that although exact conformation of the N-terminus does not influence the overall tetrameric structure or active site of the enzyme (Fig. 6B,C), the structural requirements for substrate binding and retention in the active site may depend on an intact N-terminus. Residues 23-32 of the ASL have been proposed to be important for substrate binding (50-51).

From our computational analysis we found that exon 2 deleted protein can form a stable monomer (Fig. 5B) and homotetramer (Fig. 6D), which was confirmed by western blots showing a smaller sized complex from the cell-based assays (Fig. 3C-G). However, such a homotetramer will be devoid of activity due to loss of N-terminal residues needed for substrate binding. Therefore, although an exon 2 deleted transcript of ASL is still capable of expressing a stable monomer which can form a tetrameric complex, the loss of residues involved in catalysis means such a complex can not be enzymatically active. It is possible that ASL-wt and ex2del monomers can form heterotetramers with different combinations of the two transcript variants. Since the active site of the ASL protein is formed by contributions from three different monomeric subunits, each unit of exon 2 deleted transcript variants in the heterotetramer will result in loss of one active site, without compromising the overall structure. Since the N-terminal residues affected by deletion of exon 2 do not participate in the formation of ASL dimers, we propose that a combination of wt, ex2del and wt-ex2del versions of the dimers is generated which will then further oligomerize to form the tetrameric structures. Wt and ex2del variants of the monomers may have preference for similar sized proteins and only form homodimers, which will result in at least two functional active sites in a 2:2 heterotetramer. Functional data from cell based assays which show that even 1:5 combination of wt-ex2del

ASL retained about 30% activity (Fig. 7C) seems to support this model. A combination of WT-exon2del dimers may also give similar activities.

Role of Exon 7 residues

Residues 175-200 are contributed by exon 7 in the wt ASL transcript. Exon 7 deleted variant was found to be capable of forming a monomeric structure still composed of three distinct domains, similar to that of WT ASL (Fig. 5C). While the core of homotetramer of ASL is formed by alpha helices of monomers formed by residues 291-314, we found ionic interactions between adjacent dimeric subunits in the tetramer to stabilize the structure (Fig. 6E,F). From the molecular dynamic simulations and structural analysis we found that charge pair interactions between R193 on one subunit with E189 and E185 of another adjacent subunit in the tetramer stabilizes the structure (Fig. 6G). The loss of ionic interactions contributed by exon 7 residues will result in an unstable tetramer. Computational potential energy of WT and exon7del tetramers supported this theory with an increase in potential energy for the exon7del tetramer (-76378 kJ/mol for exon7del compared to -95229 kJ/mol for WT) (Table 2). Moreover, a change in core structure of the monomers was also observed with loss of two central helices and formation of a structurally unstable loop in the middle of the helical core (Fig. 5C, 6G,H). Such a change is likely to impact the stability of the monomers and may result in an unstable protein which will likely be degraded, especially in the absence of a stable tetrameric complex. Significantly, due to this unstructured loop formation the accessible surface area of the ex7del variant increased significantly (65259 \AA^2 compared to 53534 \AA^2 for the WT) while solvent accessible surface remained similar (326476 \AA^3 compared to 322934 \AA^3 for the WT) which further points toward structural instability of ex7del variant (Table 2). This was also further supported by western blots showing no detectable ASL-ex7del expression (Fig. 3C-E,G).

Residual ASL enzymatic activities in (co-) transfected 293T cell extracts

To determine whether the expressed ASL splice variants have any residual enzyme activity, we performed ASL enzyme activity assays with the cell extracts used for Western blot analysis (summary of data in Table 1, Table 3 and Fig. 7). There was no relevant background activity in

cells transfected with the empty vector or non-transfected cells (Fig. 7A: columns 1 and 2, respectively). No significant residual activity was detected in exon 2- and exon 7-deleted ASL splice variants (Fig. 7A: columns 3 and 4, respectively) while ASL-wt transfection yielded high enzymatic activity (Fig. 7A: column 5).

To study the effect of ASL splice variants on the function of the ASL homotetramer, and the possibility of a heterotetramer formation with reduced ASL activity that may contribute to the biochemical and clinical variability in ASA patients, we measured the ASL activity after co-transfections. The residual ASL activities in cells co-expressing P-wt and P-ex2del (Fig. 7B,C: column 2) or co-expressing P-wt, P-ex2del and P-ex7del (Fig. 7B: column 4) showed a significant decrease to $62.5 \pm 7.2\%$ or $55.5 \pm 7.2\%$ (mean \pm SD), respectively, of cells co-transfected with P-wt and EV (Fig. 7B,C: column 1 and Table 1). Cells co-transfected with P-wt and P-ex7del (Fig. 7B,C: column 3) displayed no decrease of ASL activity ($94.2 \pm 9.4\%$ of ASL wt). Cells co-transfected with P-ex2del and P-ex7del (Fig. 7B: column 5) showed no relevant residual ASL activity. Furthermore, the reduced level of residual ASL activities exhibited no significant difference between cells co-expressing P-wt and P-ex2del and cells co-expressing P-wt, P-ex2del and P-ex7del (Fig. 7B: column 4). Interestingly, cells expressing only the ASL mutant P-E189G had a similar level of ASL-wt activity (Fig. 7C: column 4) but cells co-expressing P-wt and P-E189G showed two-folds levels of wt activity (Fig. 7C: column 7). However, the residual activity in cells co-transfected with P-E189G and P-ex2del or co-transfected with P-E189G and P-ex7del (Fig. 7D: column 5 or 6) exhibited a significant decrease to $16.6 \pm 5.3\%$ or $57.5 \pm 3.2\%$ (mean \pm SD), respectively, of cells co-transfected with P-wt and EV (Table 3). These findings indicate that the truncated protein caused by deletion of ASL exon 2, although showing no relevant residual activity, has a dominant negative effect on the ASL activity after co-expression with P-wt or P-E189G. In contrast, deletion of exon 7 seems to have no significant effect on ASL-wt activity after co-expression with P-wt, since it probably forms an unstable protein according to Western Blot analysis (Fig. 3C-E,G) and computational predictions (Fig. 5C, 6G-H), but has nevertheless a potential dominant negative effect on ASL mutant activity after co-expression with P-E189G (Fig. 7C: column 6).

In order to further assess whether the negative effect of the simultaneous expression of ex2del mutant on ASL-wt is dose-dependent *in vitro*, we performed transient co-transfections with P-wt and P-ex2del at different ratios 1:1, 1:2, 1:5, 2:1 or 5:1. The co-expression of ASL-wt and ASL-ex2del at above different ratios could be detected by Western Blot analysis (Fig. 3F). In cells co-expressing ASL-wt and ASL-ex2del with higher proportion of mutant DNA, we observed a significant gradual decrease of ASL-activity when compared with cells co-expressing wt and EV (Fig. 7D). Moreover, cells co-expressing ASL-wt and ASL-ex2del with higher proportion of wt DNA showed a significant gradual increase of ASL-activity (Fig. 7D). These findings indicate that the negative effect of ASL-ex2del on the wt activity is dose-dependent.

Discussion

To date, the mechanism of the broad biochemical and clinical heterogeneity in ASA patients still remains to be fully explained as it is not just the result of different genotypes. Possible hypotheses include ASL tissue specific expression (27-28), genetic variability (29) and intragenic complementation at the ASL locus (7,30-32), the DNA methylation status (35) and the frequent occurrence of alternative ASL splicing variants (5,36-37). In addition, several hormones such as glucocorticosteroids and insulin influence the regulation of ASL mRNA (52). Recently, a complex formation with the enzymes argininosuccinate synthetase and nitric oxide synthase required for nitric oxide synthesis was described (2), adding further to the complexity of this, obviously not only, urea cycle enzyme. In the present study, we were interested in the tissue-dependence of ASL transcript variants and their role for the clinical and biochemical phenotype in ASA.

RT-PCR yielded comparable expression levels for the detected transcript variants in 17 different human tissues (Fig. 2) suggesting that the expression of transcript variants is not tissue-dependent. These findings are consistent with earlier reports, in which full-length ASL expression was studied in 11 different human tissues (53) and with previous reports on the frequent skipping of exons 2 or 7 (5,36).

However, it remains pivotal to understand whether the frequent transcript variants in this gene indeed play a role in ASA patients at

physiological levels or if present at higher concentrations. To do so, we further investigated the expression levels of transcript variants as well as their enzymatic characteristics *in vitro*. Establishment of an ASL expression system in 293T cells (Fig. 3A) allowed us to investigate the ASL activities after ectopically expressing or co-expressing ASL-wt and one ASL mutant (p.Glu189Gly) or/and splicing variants. Immunoblotting showed non-detectable ASL expression in cells transfected with the ex7del construct (Fig. 3C-E,G) yielding unchanged ASL activity levels after co-expression of ASL-wt and ASL-ex7del (Fig. 7B,C and Table 1), but having a potential dominant negative effect on the ASL mutant activity after co-expression of ASL mutant p.Glu189Gly and ASL-ex7del (Fig. 7C and Table 3). Moreover, the observation of a 2-fold increase in the ASL activity after co-expressing P-wt and P-E189G (Fig. 7C: column 7) further supports the view that the negative effect on ASL mutant activity in cells co-transfected with P-E189G and P-ex7del is not caused by the mutant itself. Computational structure modelling revealed that ex7del variant was capable to form a monomeric structure with three distinct domains, similar to that of ASL-wt (Fig. 5C). As predicted by structural analysis, ex7del may interfere with tetramer formation and seriously impact the stability of the core monomeric structure (Fig. 5), leading to premature degradation of the monomers. This may explain the undetectable ASL-exdel7 monomers after co-expression with ASL-wt or with p.E189G (Fig. 3D,G). Nevertheless, the ex7del ASL monomer may still form a heterotetramer with other monomers before degradation occurs, especially with easily accessible mutants such as p.Glu189Gly, which then would result in reduced ASL activity. It should be noted that exon 7 contains 78 bases allowing formation of an in-frame mutant ASL protein (5). This finding is somewhat different to an earlier study (54), in which a small amount of a 49-kDa band in addition to the expected ASL-wt band on Western blots was speculated as exon 7 skipping product that may be due to proteolysis (5). However, the details of the possible degradation of ex7del ASL were not investigated in this study.

Although exon 2 skipping was observed in previous studies (29,36), these authors didn't determine the molecular basis and impact for this deletion. As one of the main findings of our investigations, we could show that exon 2-spliced ASL can form a stable truncated protein

(Fig. 3C-G) but it lacks any relevant activity (Fig. 7A; Table 1). This finding is consistent with our prediction explored by computational structure modelling that exon 2 is in close vicinity to the active site (Fig. 6B,C) and its deletion is probably deleterious. Surprisingly, this stable truncated mutant protein has a dominant negative effect on the ASL activity after co-expressing ASL-wt as well as ASL-p.Glu189Gly with ASL-ex2del (Fig. 7B,C); in the latter case, this was even more relevant. Furthermore, this negative effect is ASL-ex2del DNA dose-dependent (Fig. 7D). The reduced ASL activity may result from the formation of a heterotetrameric structure between ASL-wt and ASL-ex2del mutant protein as well as homotetrameric ex2del variants that are enzymatically inactive, competing for the substrate, depending on their proportions. Since three different subunits of ASL contribute to the active site in the tetrameric structures, different combinations of wt and ex2del variant subunits may combine to form tetramers with 3, 2, 1 or 0 active sites. It is possible that proteins of different size resulting from wt or exon 2 deleted transcripts may have a preference for each other when forming the dimeric units before joining to form tetramers. In such a scenario only 2:2 versions of the heterotetramers may be the preferred assembly in addition to the fully functional and non-functional molecules with 4 or 0 active sites. Due to a lack of liver samples derived from ASA patients, we could not show whether a different amount of ASL-ex2del mutant protein is expressed in the ASA patients, which may interact with ASL-wt to form a heterotetramer leading to different level of residual ASL activity.

While this study clearly shows that transcript variants under physiological conditions are only present at low concentrations (Fig. 2A-D), the situation was found different in two patients affected by late onset ASA (Fig. 2C: lanes 2 and 3; Fig. 1D: lane 16). Both patients were shown to carry the same known ASL missense mutation (p.Glu189Gly) with low residual ASL activity in red blood cells found in one of the patients (25),

absent activity after bacterial overexpression (33) but significant residual activity in yeast experiments (55). While the mutation was found in a heterozygous state by genomic DNA sequencing, the level of the ASL-ex7del transcript variant observed by RNA studies was much higher (Fig 2C: lanes 2 and 3; Fig. 2D: lane 16). Under standard PCR conditions (38 cycles), no full-length ASL transcript was amplified, but a faint full-length band was detected after 42 PCR cycles indicating the low expression of this transcript. Sequencing confirmed the mutation c.566A>G in a hemizygous state in one patient (Fig. 2E) suggesting that the ASL wt allele in this particular patient is completely subject to alternative splicing and hence, no wt transcript is expressed while in the other patient heterozygosity was found (Fig. 2F). We suggest here that the high expression level of the ASL-ex7del variant contributed to the ASA phenotype in the patients in addition to the missense mutation. From the finding in these patients we deduce that the same phenomenon may play a role also in other patients possibly affected by any of the transcript variants (such as patient 16 in (25) with no mutation found but skipping of exon 5).

In conclusion, this study suggests that transcript variants of ASL exist at high frequency in different tissues and play a role for the clinical and biochemical variability in ASA patients in whom they are expressed at high levels. If this occurs with an increased expression level of a stable expressed truncated variant such as ASL-ex2del variant, the effect may be even more prominent since the likelihood of stable mutant homo- or heterotetramer formation increases impairing overall ASL activity. Although the unstable splice variant ASL-ex7del has no effect on ASL-wt activity, it shows likely a dominant negative effect on ASL mutant activity. The findings from this study expand our knowledge of the molecular background in ASA patients rendering further studies into transcript variants necessary, especially in patients with no or only single mutations.

References

1. Brusilow, S., and Horwich, A. (2001) Urea cycle enzymes. in *The metabolic & molecular bases of inherited disease* (Scriver, C., Beaudet, A., Sly, W., and Valle, D. eds.), 8th Ed., McGraw-Hill, New York. pp 1909-1963
2. Erez, A., Nagamani, S. C., Shchelochkov, O. A., Premkumar, M. H., Campeau, P. M., Chen, Y., Garg, H. K., Li, L., Mian, A., Bertin, T. K., Black, J. O., Zeng, H., Tang,

- Y., Reddy, A. K., Summar, M., O'Brien, W. E., Harrison, D. G., Mitch, W. E., Marini, J. C., Aschner, J. L., Bryan, N. S., and Lee, B. (2011) Requirement of argininosuccinate lyase for systemic nitric oxide production. *Nature medicine* **17**, 1619-1626
3. Todd, S., McGill, J. R., McCombs, J. L., Moore, C. M., Weider, I., and Naylor, S. L. (1989) cDNA sequence, interspecies comparison, and gene mapping analysis of argininosuccinate lyase. *Genomics* **4**, 53-59
4. Naylor, S. L., Klebe, R. J., and Shows, T. B. (1978) Argininosuccinic aciduria: assignment of the argininosuccinate lyase gene to the pter to q22 region of human chromosome 7 by bioautography. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 6159-6162
5. Abramson, R. D., Barbosa, P., Kalumuck, K., and O'Brien, W. E. (1991) Characterization of the human argininosuccinate lyase gene and analysis of exon skipping. *Genomics* **10**, 126-132
6. Linnebank, M., Tschiedel, E., Häberle, J., Linnebank, A., Willenbring, H., Kleijer, W. J., and Koch, H. G. (2002) Argininosuccinate lyase (ASL) deficiency: mutation analysis in 27 patients and a completed structure of the human ASL gene. *Human genetics* **111**, 350-359
7. Turner, M. A., Simpson, A., McInnes, R. R., and Howell, P. L. (1997) Human argininosuccinate lyase: a structural basis for intragenic complementation. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 9063-9068
8. Matsubasa, T., Takiguchi, M., Amaya, Y., Matsuda, I., and Mori, M. (1989) Structure of the rat argininosuccinate lyase gene: close similarity to chicken delta-crystallin genes. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 592-596
9. Piatigorsky, J., O'Brien, W. E., Norman, B. L., Kalumuck, K., Wistow, G. J., Borrás, T., Nickerson, J. M., and Wawrousek, E. F. (1988) Gene sharing by delta-crystallin and argininosuccinate lyase. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 3479-3483
10. Piatigorsky, J., and Horwitz, J. (1996) Characterization and enzyme activity of argininosuccinate lyase/delta-crystallin of the embryonic duck lens. *Biochim Biophys Acta* **1295**, 158-164
11. Ratner, S. (1973) Enzymes of arginine and urea synthesis. *Adv Enzymol Relat Areas Mol Biol* **39**, 1-90
12. Ratner, S., and Petrack, B. (1953) The mechanism of arginine synthesis from citrulline in kidney. *The Journal of biological chemistry* **200**, 175-185
13. De Jonge, W. J., Dingemanse, M. A., de Boer, P. A., Lamers, W. H., and Moorman, A. F. (1998) Arginine-metabolizing enzymes in the developing rat small intestine. *Pediatric research* **43**, 442-451
14. Flynn, N. E., Meininger, C. J., Kelly, K., Ing, N. H., Morris, S. M., Jr., and Wu, G. (1999) Glucocorticoids mediate the enhanced expression of intestinal type II arginase and argininosuccinate lyase in postweaning pigs. *The Journal of nutrition* **129**, 799-803
15. Walker, J. B. (1958) Role for pancreas in biosynthesis of creatine. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y)* **98**, 7-9
16. Pisarenko, S. I., Minkovskii, E. B., and Studneva, I. M. (1980) [Urea synthesis in the myocardium]. *Biulleten' eksperimental'noi biologii i meditsiny* **89**, 165-168
17. Ratner, S., Morell, H., and Carvalho, E. (1960) Enzymes of arginine metabolism in brain. *Archives of biochemistry and biophysics* **91**, 280-289

18. Jones, M. E., Anderson, A. D., Anderson, C., and Hodes, S. (1961) Citrulline synthesis in rat tissues. *Archives of biochemistry and biophysics* **95**, 499-507
19. O'Brien, W. E., and Barr, R. H. (1981) Argininosuccinate lyase: purification and characterization from human liver. *Biochemistry* **20**, 2056-2060
20. Tomlinson, S., and Westall, R. G. (1964) Argininosuccinic Aciduria. Argininosuccinase and Arginase in Human Blood Cells. *Clinical science* **26**, 261-269
21. Allan, J. D., Cusworth, D. C., Dent, C. E., and Wilson, V. K. (1958) A disease, probably hereditary characterised by severe mental deficiency and a constant gross abnormality of aminoacid metabolism. *Lancet* **1**, 182-187
22. Brusilow, S. W., and Maestri, N. E. (1996) Urea cycle disorders: diagnosis, pathophysiology, and therapy. *Advances in pediatrics* **43**, 127-170
23. Wong, L. T., Hardwick, D. F., Applegarth, D. A., and Davidson, A. G. (1979) Review of Metabolic Screening Program of Children's Hospital, Vancouver, British Columbia. 1971--1977. *Clinical biochemistry* **12**, 167-172
24. Ficicioglu, C., Mandell, R., and Shih, V. E. (2009) Argininosuccinate lyase deficiency: longterm outcome of 13 patients detected by newborn screening. *Molecular genetics and metabolism* **98**, 273-277
25. Mercimek-Mahmutoglu, S., Moeslinger, D., Häberle, J., Engel, K., Herle, M., Strobl, M. W., Scheibenreiter, S., Muehl, A., and Stockler-Ipsiroglu, S. (2010) Long-term outcome of patients with argininosuccinate lyase deficiency diagnosed by newborn screening in Austria. *Molecular genetics and metabolism* **100**, 24-28
26. Erez, A., Nagamani, S. C., and Lee, B. (2011) Argininosuccinate lyase deficiency-argininosuccinic aciduria and beyond. *Am J Med Genet C Semin Med Genet* **157**, 45-53
27. Glick, N. R., Snodgrass, P. J., and Schafer, I. A. (1976) Neonatal argininosuccinic aciduria with normal brain and kidney but absent liver argininosuccinate lyase activity. *American journal of human genetics* **28**, 22-30
28. Perry, T. L., Wirtz, M. L., Kennaway, N. G., Hsia, Y. E., Atienza, F. C., and Uemura, H. S. (1980) Amino acid and enzyme studies of brain and other tissues in an infant with argininosuccinic aciduria. *Clinica chimica acta; international journal of clinical chemistry* **105**, 257-267
29. Barbosa, P., Cialkowski, M., and O'Brien, W. E. (1991) Analysis of naturally occurring and site-directed mutations in the argininosuccinate lyase gene. *The Journal of biological chemistry* **266**, 5286-5290
30. Howell, P. L., Turner, M. A., Christodoulou, J., Walker, D. C., Craig, H. J., Simard, L. R., Ploder, L., and McInnes, R. R. (1998) Intragenic complementation at the argininosuccinate lyase locus: reconstruction of the active site. *Journal of inherited metabolic disease* **21 Suppl 1**, 72-85
31. McInnes, R. R., Shih, V., and Chilton, S. (1984) Interallelic complementation in an inborn error of metabolism: genetic heterogeneity in argininosuccinate lyase deficiency. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 4480-4484
32. Walker, D. C., Christodoulou, J., Craig, H. J., Simard, L. R., Ploder, L., Howell, P. L., and McInnes, R. R. (1997) Intragenic complementation at the human argininosuccinate lyase locus. Identification of the major complementing alleles. *The Journal of biological chemistry* **272**, 6777-6783
33. Engel, K., Vuissoz, J. M., Eggimann, S., Groux, M., Berning, C., Hu, L., Klaus, V., Moeslinger, D., Mercimek-Mahmutoglu, S., Stockler, S., Wermuth, B., Häberle, J., and Nuoffer, J. M. (2012) Bacterial expression of mutant argininosuccinate lyase reveals imperfect correlation of in-vitro enzyme activity with clinical phenotype in argininosuccinic aciduria. *Journal of inherited metabolic disease* **35**, 133-140

34. Trevisson, E., Burlina, A., Doimo, M., Pertegato, V., Casarin, A., Cesaro, L., Navas, P., Basso, G., Sartori, G., and Salviati, L. (2009) Functional complementation in yeast allows molecular characterization of missense argininosuccinate lyase mutations. *The Journal of biological chemistry* **284**, 28926-28934
35. Renouf, S., Fairand, A., and Husson, A. (1998) Developmental control of argininosuccinate lyase gene by methylation. *Biology of the neonate* **73**, 190-197
36. Linnebank, M., Homberger, A., Rapp, B., Winter, C., Marquardt, T., Harms, E., and Koch, H. G. (2000) Two novel mutations (E86A, R113W) in argininosuccinate lyase deficiency and evidence for highly variable splicing of the human argininosuccinate lyase gene. *Journal of inherited metabolic disease* **23**, 308-312
37. Walker, D. C., McCloskey, D. A., Simard, L. R., and McInnes, R. R. (1990) Molecular analysis of human argininosuccinate lyase: mutant characterization and alternative splicing of the coding region. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 9625-9629
38. Landsverk, M. L., Wang, J., Schmitt, E. S., Pursley, A. N., and Wong, L. J. (2011) Utilization of targeted array comparative genomic hybridization, MitoMet, in prenatal diagnosis of metabolic disorders. *Molecular genetics and metabolism* **103**, 148-152
39. Kretz, R., Hu, L., Wettstein, V., Leiteritz, D., and Haberle, J. (2012) Phytohemagglutinin stimulation of lymphocytes improves mutation analysis of carbamoylphosphate synthetase 1. *Molecular genetics and metabolism* **106**, 375-378
40. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *The Journal of biological chemistry* **193**, 265-275
41. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
42. Krieger, E., Darden, T., Nabuurs, S. B., Finkelstein, A., and Vriend, G. (2004) Making optimal use of empirical energy functions: force-field parameterization in crystal space. *Proteins* **57**, 678-683
43. Vriend, G. (1990) WHAT IF: a molecular modeling and drug design program. *J Mol Graph* **8**, 52-56, 29
44. Hooft, R. W., Vriend, G., Sander, C., and Abola, E. E. (1996) Errors in protein structures. *Nature* **381**, 272
45. Bowie, J. U., Luthy, R., and Eisenberg, D. (1991) A method to identify protein sequences that fold into a known three-dimensional structure. *Science* **253**, 164-170
46. Luthy, R., Bowie, J. U., and Eisenberg, D. (1992) Assessment of protein models with three-dimensional profiles. *Nature* **356**, 83-85
47. Ramachandran, G. N., Ramakrishnan, C., and Sasisekharan, V. (1963) Stereochemistry of polypeptide chain configurations. *J Mol Biol* **7**, 95-99
48. Hooft, R. W., Sander, C., and Vriend, G. (1997) Objectively judging the quality of a protein structure from a Ramachandran plot. *Comput Appl Biosci* **13**, 425-430
49. Liu, H., Elstner, M., Kaxiras, E., Frauenheim, T., Hermans, J., and Yang, W. (2001) Quantum mechanics simulation of protein dynamics on long timescale. *Proteins* **44**, 484-489
50. Vallee, F., Turner, M. A., Lindley, P. L., and Howell, P. L. (1999) Crystal structure of an inactive duck delta II crystallin mutant with bound argininosuccinate. *Biochemistry* **38**, 2425-2434
51. Sampaleanu, L. M., Yu, B., and Howell, P. L. (2002) Mutational analysis of duck delta 2 crystallin and the structure of an inactive mutant with bound substrate provide insight into the enzymatic mechanism of argininosuccinate lyase. *The Journal of biological chemistry* **277**, 4166-4175

52. Husson, A., Renouf, S., Fairand, A., Buquet, C., Benamar, M., and Vaillant, R. (1990) Expression of argininosuccinate lyase mRNA in foetal hepatocytes. Regulation by glucocorticoids and insulin. *European journal of biochemistry / FEBS* **192**, 677-681
53. Neill, M. A., Aschner, J., Barr, F., and Summar, M. L. (2009) Quantitative RT-PCR comparison of the urea and nitric oxide cycle gene transcripts in adult human tissues. *Molecular genetics and metabolism* **97**, 121-127
54. Simard, L., O'Brien, W. E., and McInnes, R. R. (1986) Argininosuccinate lyase deficiency: evidence for heterogeneous structural gene mutations by immunoblotting. *American journal of human genetics* **39**, 38-51
55. Doimo, M., Trevisson, E., Sartori, G., Burlina, A., and Salvati, L. (2012) Yeast complementation is sufficiently sensitive to detect the residual activity of ASL alleles associated with mild forms of argininosuccinic aciduria. *Journal of inherited metabolic disease* **35**, 557-558

Footnotes

¹*Acknowledgments*-The authors would like to acknowledge the technical assistance provided by Dana Leiteritz, Zurich. We thank Drs. C. Giunta and D. Coelho (both Zurich) for their helpful suggestions during planning of experiments. We also would like to acknowledge Dr. Lee-Jun C. Wong (Baylor College of Medicine, Houston USA) for performing array comparative genomic hybridization.

²This work was supported by the Swiss National Science Foundation [grants No. 310030_127184/1 to JH and 31003A-134926 to AVP] and a grant from Schweizerische Mobiliar Genossenschaft Jubiläumsstiftung to AVP.

³*Conflict of interest*-The authors of this paper confirm that there is no conflict of interest.

⁴*Abbreviations*-ASA, argininosuccinic aciduria; ASL, argininosuccinate lyase; EV, empty vector pcDNA3; NT, non-transfected 293T cells; P-E189G, pcDNA3-ASL-Glu189Gly; P-wt, pcDNA3-ASL-wt; ex2del, exon 2-deleted; P-ex2del, pcDNA3-ASL-ex2del; ex7del, exon 7-deleted; P-ex7del, pcDNA3-ASL-ex7del; wt, wildtype

Figure legends

Figure 1: Involvement of argininosuccinate lyase in various metabolic and biochemical pathways

Schematic illustration of the involvement of ASL in various metabolic and biochemical reactions. Colours are used to show the affiliation of metabolites and enzymes to different pathways: blue, urea cycle; golden, nitric oxide synthesis; bordeaux, polyamine synthesis; purple, creatine synthesis. Enzymes are depicted in ovals: ADC, arginine decarboxylase; AGAT, arginine:glycine amidinotransferase; ARG, arginase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS1, carbamoylphosphate synthetase 1; GAMT, guanidinoacetate methyltransferase; NOS, nitric oxide synthase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase.

Figure 2: Occurrence of transcript variants of the ASL gene in different tissues

PCR products representing short fragments of ASL cDNA from 16 different tissues (panel A), from cultured skin fibroblasts derived from 12 ASA patients (panel B), from lymphocytes derived from two ASA patients and one patient's mother (panel C) and from controls' lymphocytes, livers and fibroblasts as well as lymphocytes derived from one ASA patient and his mother (panel D). **A:** lane 1-16: 1, skin fibroblast; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, peripheral leukocytes. **B:** lane 1-12 (obtained from three different investigations): skin fibroblasts from 12 ASA patients with 10 different genotypes. **C:** lane 1-4: 1, mother of patient in lane 2; 2-3, two ASA patients; 4, control. **D:** lane 1-15: 5 controls' lymphocytes (lane 1-5), livers (lane 6-10) and fibroblasts (lane 11-15); lane 16: lymphocytes from one patient (same patient as in Fig. 2C lane 2); lane 17: lymphocytes from mother of patient in lane 16 (same as in Fig. 2C lane 1). **E:** Sequencing

chromatogram of PCR product from lane 2 in Fig. 2C. **F:** Sequencing chromatogram of PCR product from lane 3 in Fig. 2C. M: pGOLD 100 bp DNA ladder plus (Peqlab, Erlangen, Germany); M': 100 bp DNA ladder (Solis BioDyne, Tartu, Estonia). Gel electrophoresis was done in 1% agarose.

Figure 3: Expression or co-expression of ASL wildtype and transcript variants in different mammalian cell lines and human livers

Western blot analysis of ASL expression in cell extracts of COS-1, 293T and HeLa cells (A), in human livers (B) or in transfected 293T cells (C-G). 30 µg of total protein was separated by 10% SDS-PAGE (A-B, D, F-G and upper panel in C) or by native PAGE (E and middle panel in C) for analysing ASL expression. **A:** NT, non-transfected; EV, empty vector pcDNA3; wt, pcDNA3-ASL-wt (P-wt). **B:** lane 1-3: human liver samples from 3 controls. **C:** ASL expression in 293T cells non-transfected (NT) or transfected with EV, P-wt, pcDNA3-ASL-ex2del (P-ex2del) or pcDNA3-ASL-ex7del (P-ex7del), respectively. **D:** (Co-)expression of ASL monomers in 10% SDS-PAGE (ASL-wt: 52 kDa, ASL-ex2del: 45 kDa). Lane 1-7: 1, P-wt with EV; 2, P-wt with P-ex2del and EV; 3, P-wt with P-ex7del and EV; 4, P-wt with P-ex2del and P-ex7del; 5, P-ex2del; 6, P-ex7del; 7, EV. **E:** (Co-) expression of ASL homotetramers in 10% native PAGE (ASL-wt: ~200 kDa, ASL-ex2del: ~180 kDa). Lane 1-7 same as in D. Asterisks indicate unspecific bands. **F:** ASL expression in 293T cells co-transfected with P-wt and P-ex2del at different ratios. Lane 1-9: 1, P-wt (1.75 µg); 2, 1:1; 3, 1:2; 4, 1:5; 5, P-wt (3.5 µg); 6, 2:1; 7, P-wt (8.75 µg); 8, 5:1; 9, ASL-ex2del (3.5 µg). **G:** (Co-)expression of ASL mutant p.Glu189Gly (P-E189G) with transcript variants. Lane 1-9: 1, P-wt with EV; 2, P-E189G with EV; 3, P-wt with P-E189G; 4, P-wt with P-ex2del; 5, P-wt with P-ex7del; 6, P-E189G with P-ex2del; 7, P-E189G with P-ex7del; 8, P-ex2del; 9, P-ex7del. Asterisks indicate unspecific bands. β-actin (42 kDa) and GAPDH (37 kDa) served as loading control.

Figure 4: Positions of exons 2 and 7 in secondary structure of the ASL protein

The helices are in yellow, sheets in green and turns in cyan; exons 2 and 7 are depicted in magenta. Amino acids are colored based on their chemical properties with aspartic and glutamic acid in red, arginines and lysines in blue and aromatic amino acids in green.

Figure 5: Monomeric structure of wildtype and models of ASL exon 2- or exon 7-deleted transcript variant

A: Monomeric structure of wt ASL. ASL monomer has three distinct subdomains, domains 1 and 3 have similar structure and topology with two helix-turn-helix motifs in perpendicular arrangement. Domain 2 has nine helices and five of them form the central five helix bundle with up-down-up-down-up topology. **B:** Monomeric structural model of ex2del ASL. Two critical helices that are part of domain 1 (indicated by black arrow) and contribute to the active site are missing in the ex2del variant of ASL. **C:** Monomeric structural model of ex7del ASL. Exon 7 residues are part of domain 2 in the ASL monomer comprising the central five helix bundle. Deletion of exon 7 results in a disordered central core with one of the five central helices partially replaced by an unstructured loop (indicated by black arrow). N, N-terminus; C, C-terminus.

Figure 6: Tetrameric structure of wildtype and models of ASL exon 2- or exon 7-deleted transcript variant

A: Tetrameric structure of wt ASL showing the positions of active site histidine 160 and exons 2 and 7. Structure is shown as a ribbons model in light blue, amino acids contributed by exon 2 are colored in red, while amino acids contributed by exon 7 are colored in magenta. The active site residues are colored in green with H160 at the catalytic center shown a sphere model. **B:** Location of exon 2 in relation to active site. In the ASL homotetramer active site residues are contributed by three different subunits. ASL structure is shown as a ribbons model with different subunits colored in green, cyan, magenta and yellow. The green and cyan subunits form a dimer and join with another dimer formed by magenta and yellow subunits to form the tetramer. Active site residues from one of the sites are shown as solid surfaces while catalytic center (H160) is shown as spheres. Residues contributed by exon 2 are shown in red. **C:** The role of N-terminus tail in stabilizing the active site. The ASL homotetramer is shown as a solid surface model with different subunits colored in green, cyan, magenta and yellow. Residues contributed by exon 2 are shown in red. The N-terminus tail surrounds the active site and provides the stability required for binding of substrate. **D:** Conjectural prediction of

exon2 deleted ASL based on homology modelling. The core structure of ASL homotetramer can still be formed in the absence of N-terminus residues contributed by exon 2. The model of exon2del tetrameric complex is similar to the wt ASL and central four-helix bundle comprising the tetramer remain intact, resulting in a stable structure. However, the loss of N-terminus required for substrate binding will result in an enzymatically inactive protein. **E:** The wt ASL tetramer structure showing the locations of residues contributed by exon 7. The homotetramer structure is shown as a ribbons model with different subunits colored in green, cyan, magenta and yellow. The amino acids contributed by exon 7 are shown as surface models in two of the adjacent dimeric structures that form the tetramer. Chains shown in green and yellow are from two different dimers that join together to form the tetramer. The H160 residue at the catalytic centre is shown as spheres. **F:** A spacefilled surface model of homotetrameric ASL structure showing the role of exon 7 in tetramer formation. Interactions between charged residues on adjacent dimeric ASL subunits stabilize the tetrameric structure. **G:** A structural model of exon 7 deleted variant of ASL transcript. One subunit of exon 7 deleted variant is superimposed on the wt ASL homotetramer to show the structural differences. The protein is shown as ribbons model with exon 7 deleted variant colored in grey, while wt subunits in tetramer are colored in green, cyan, magenta and yellow. The residues involved in charge based interaction located on two adjacent subunits in the wt protein are shown as sticks in the larger oval red dotted box. The secondary structure elements altered in the exon 7 deleted variant are marked in smaller oval box. **H:** A closeup of the structural changes resulting from the exon 7 deletion. Exon 7 deleted variant (grey) and wt (green) are superimposed and residues contributed by exon 7 in the wt protein are shown in red. Two of the central helices in the wt protein are altered to flexible loops in the exon 7 deleted variants, resulting in an unstable protein due to requirement of core helices in the domain 2 of the ASL monomers for the overall stability. The resultant protein was found to be highly unstable and unlikely to participate in tetramer formation.

Figure 7: Analysis of recombinant ASL activities

ASL enzymatic activity analysis in non-transfected or (co)-transfected 293T cell extracts. Each 4.8-14 µg total protein of cell extracts was used for the enzyme assay. The residual ASL activities are represented in percentage of ASL-wt activity (% of ASL-wt or % of ASL-wt with EV). **A:** 293T cells were transiently transfected with 7 µg of P-wt (wt), P-ex2del (ex2del) or P-ex7del (ex7del), respectively. NT: non-transfected; EV: empty vector. Non-transfected cells and those transfected with EV served as negative control. **B:** 293T cells were transiently co-transfected with 10.5 µg of total plasmids at a ratio 1:1 (each 3.5 µg) of P-wt to P-ex2del or P-ex7del plasmids, respectively. **C:** 293T cells were transiently co-transfected with 7 µg of total plasmids at a ratio 1:1 (each 3.5 µg) of P-wt to P-ex2del or P-ex7del and of P-E189G to P-ex2del or P-ex7del plasmids, respectively. **D:** ASL enzymatic activity in 293T cell extracts co-transfected with P-wt and P-ex2del at different ratios. 3-12 µg of cell extracts was used for ASL enzyme analysis depending on the amount of P-wt used for the co-transfections. For co-transfections different ratios of ASL-wt to ex2del splice variant (1:1, 1:2, 1:5, 2:1 or 5:1) were used, respectively. The residual activity of each co-transfectant compared with ASL-wt under the same condition is indicated in percentage of ASL-wt activity (% of ASL-wt co-transfected with EV to use identical amounts of total plasmids and of P-wt for all experiments). EV was used to set up the same amount of total plasmids for the co-transfections. Levels of significance are given as p-values obtained by One-way ANOVA using GraphPad software from triplicate measurements of at least 3 independent experiments, respectively. Differences were considered as significant if p value was < 0.05.

Table 1: Residual ASL activities in 293T cells (co-)transfected with recombinant ASL-wt and transcript variants

293T whole cell extracts	NT	EV	ex2del	ex7del	wt	wt+EV	wt+EV+ex2del	wt+EV+ex7del	wt+ex2del+ex7del	ex2del+ex7del+EV
ASL activity in mIE/mg total protein (mean±SD; in triplicate)	1.2 ± 1.9	1.6 ± 2.2	3.2 ± 3.0	1.9 ± 1.4	830.2 ± 78.8	520.1 ± 297.5	307.5 ± 151.4	466.1 ± 232.7	305.3 ± 201.7	3.2 ± 1.5
ASL activity in % of ASL-wt (mean±SD; in triplicate)	0.4 ± 0.4	0.3 ± 0.2	0.5 ± 0.4	0.4 ± 0.2	100.0 ± 7.8	100.0 ± 2.6	62.5* ± 7.2	94.2 ± 9.4	55.5* ± 7.2	0.7 ± 0.3

Data were obtained under standard conditions (13.6 mM argininosuccinate) after at least three independent experiments. ASL activity in cells (co-)expressing ASL-wt or ASL-wt with EV is set to 100% in each transfection (7 µg of plasmid) or co-transfection (total 10.5 µg of plasmids, each 3.5 µg) under same condition, respectively. Significant differences compared to ASL-wt activity are indicated by an asterisk (p<0.05). NT: non-transfected cells; EV: empty vector.

Table 2: Computational determination of structural properties of ASL-wt, ASL-ex2del and ASL-ex7del proteins

	ASL-wt	ASL-ex2del	ASL-ex7del
Molecular weight	51658 Da	44471 Da	48733 Da
Theoretical pI	6.04	5.90	5.76
Solvent accessible surface	322934 Å ³	296898 Å ³	326476 Å ³
Accessible surface area	53534 Å ²	55904 Å ²	65259 Å ²
Potential energy	-95229 kJ/mol	-77219 kJ/mol	-76378 kJ/mol
Radius of gyration	36.3 Å	36.1 Å	36.9 Å
Electrostatic solvation energy	-29769 kJ/mol	-24454 kJ/mol	-27781 kJ/mol
Electrostatic potential	-14.89 kJ/mol	-20.4 kJ/mol	-43.07 kJ/mol

Computational parameters were determined with WHATIF and YASARA.

Table 3: Residual ASL activities in 293T cells co-transfected with recombinant ASL-wt, mutant p.E189G and transcript variants

293T whole cell extracts	wt + EV	wt+ex2del	wt+ex7del	E189G+EV	E189G+ex2del	E189G+ex7del	wt+E189G
mean ASL activity in mIE/mg total protein (mean±SD)	450.2 ± 38.3	286.1 ± 5.5	363.5 ± 15.0	389.1 ± 30.8	78.4 ± 26.4	257.2 ± 10.5	883.8 ± 80.0
mean ASL activity in % of ASL-wt+EV (mean±SD)	100.0 ± 7.1	61.9* ± 5.8	88.5 ± 11.5	88.4 ± 11.9	16.6* ± 5.3	57.5* ± 3.2	197.6 ± 8.9

Data were obtained under standard conditions (13.6 mM argininosuccinate) after three independent experiments. ASL activity in cells co-expressing ASL-wt with EV is set to 100% in each co-transfection (total 7 µg of plasmids, each 3.5 µg) under same condition. Significant differences compared to ASL-wt activity are indicated by an asterisk (p<0.05). All measurements in triplicate.

Figure 1

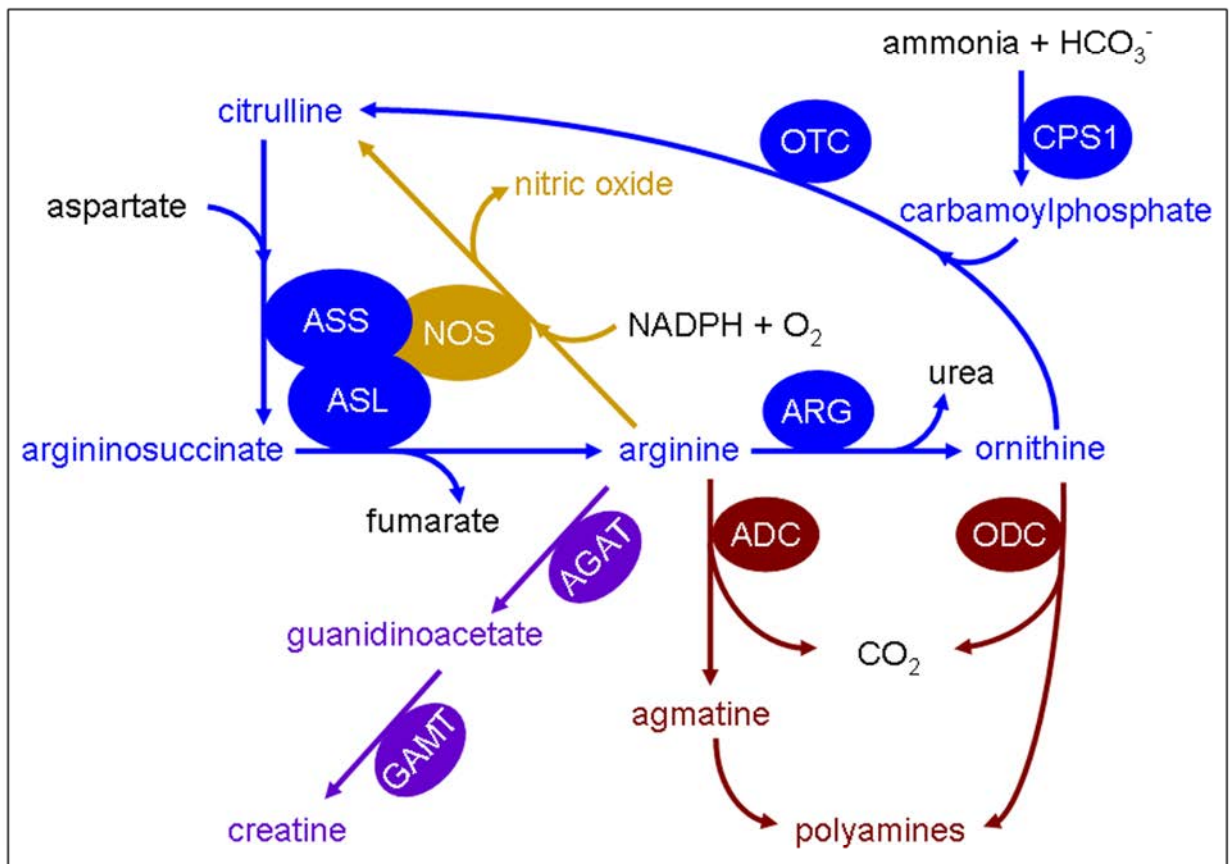


Figure 2

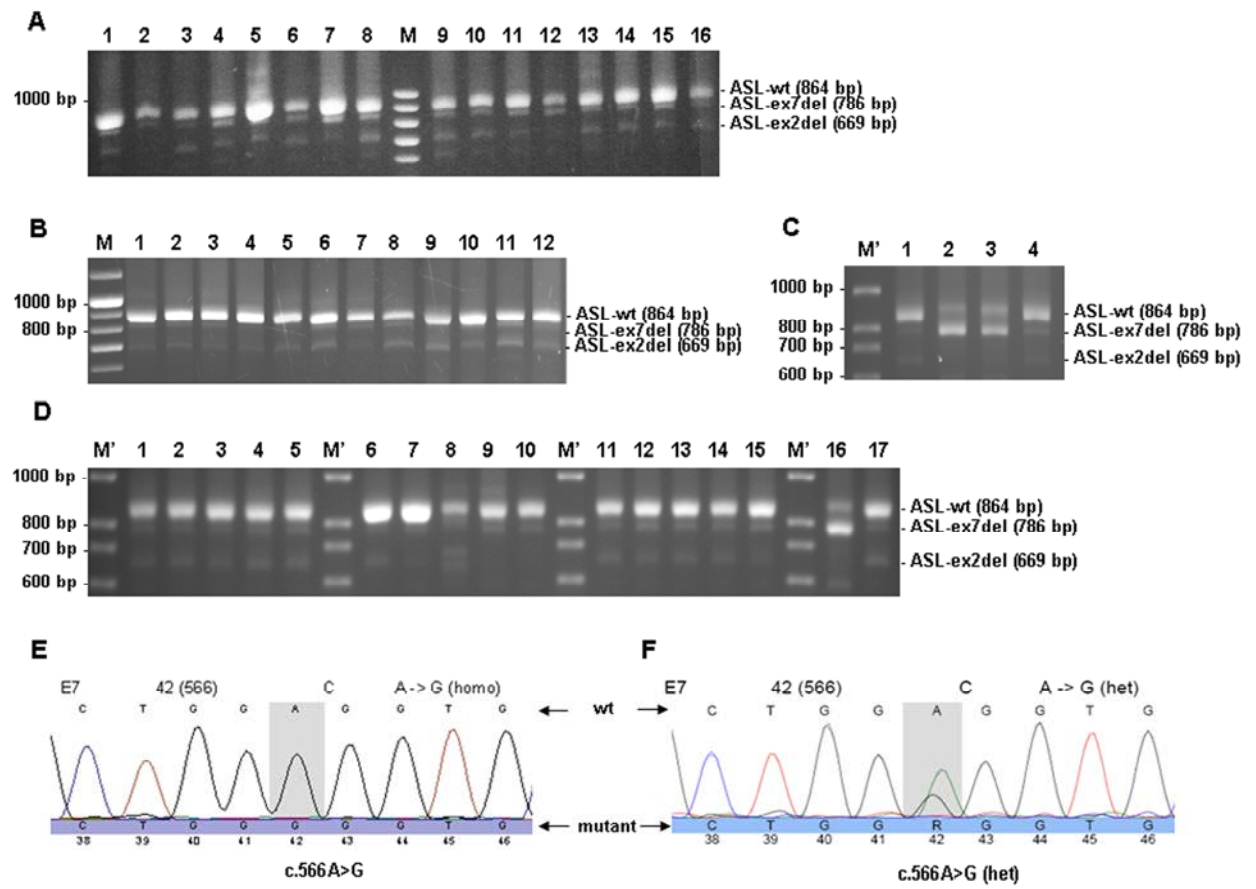


Figure 3

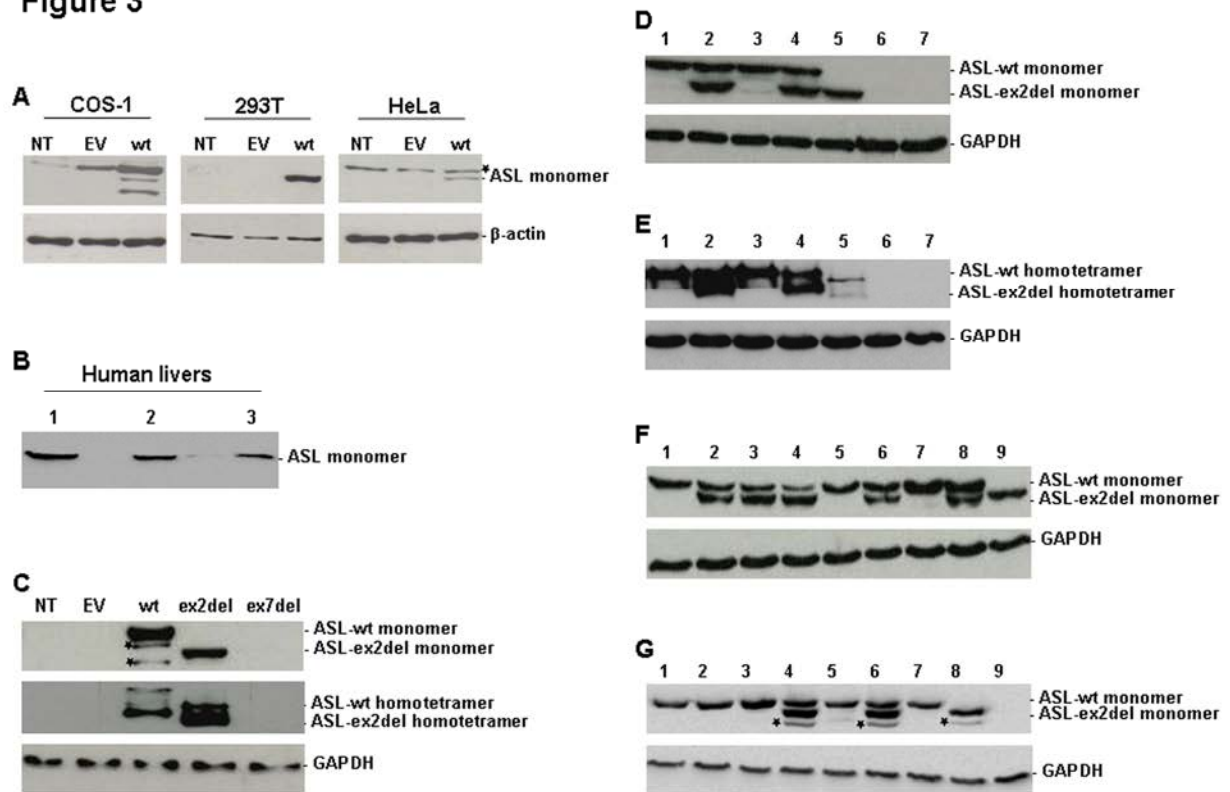


Figure 4

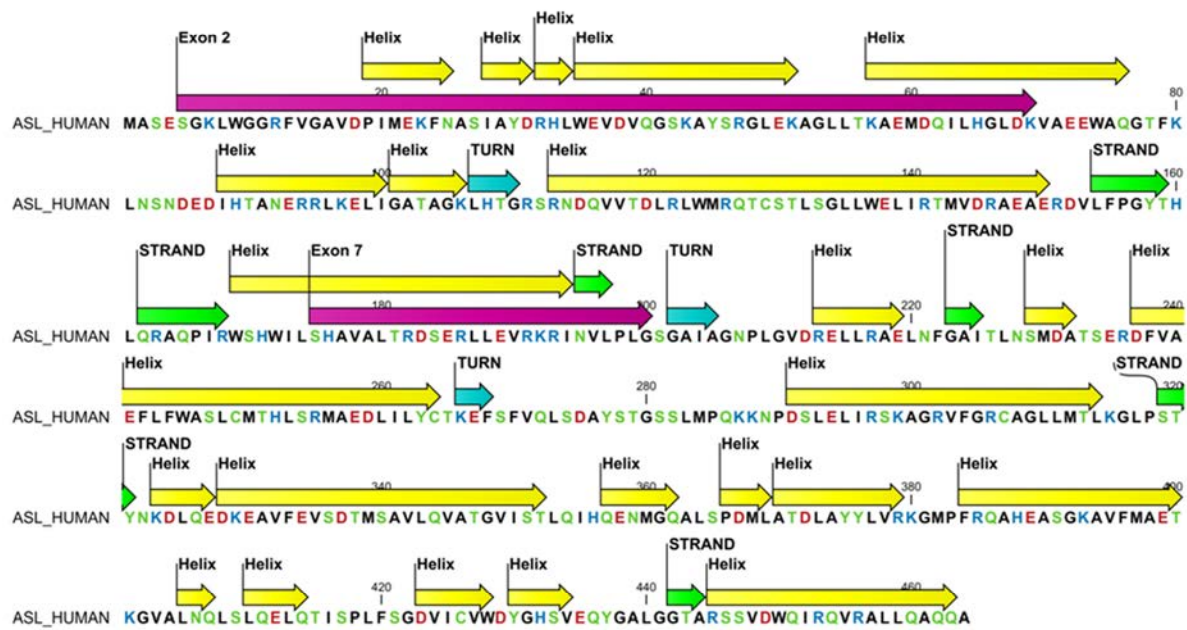


Figure 5

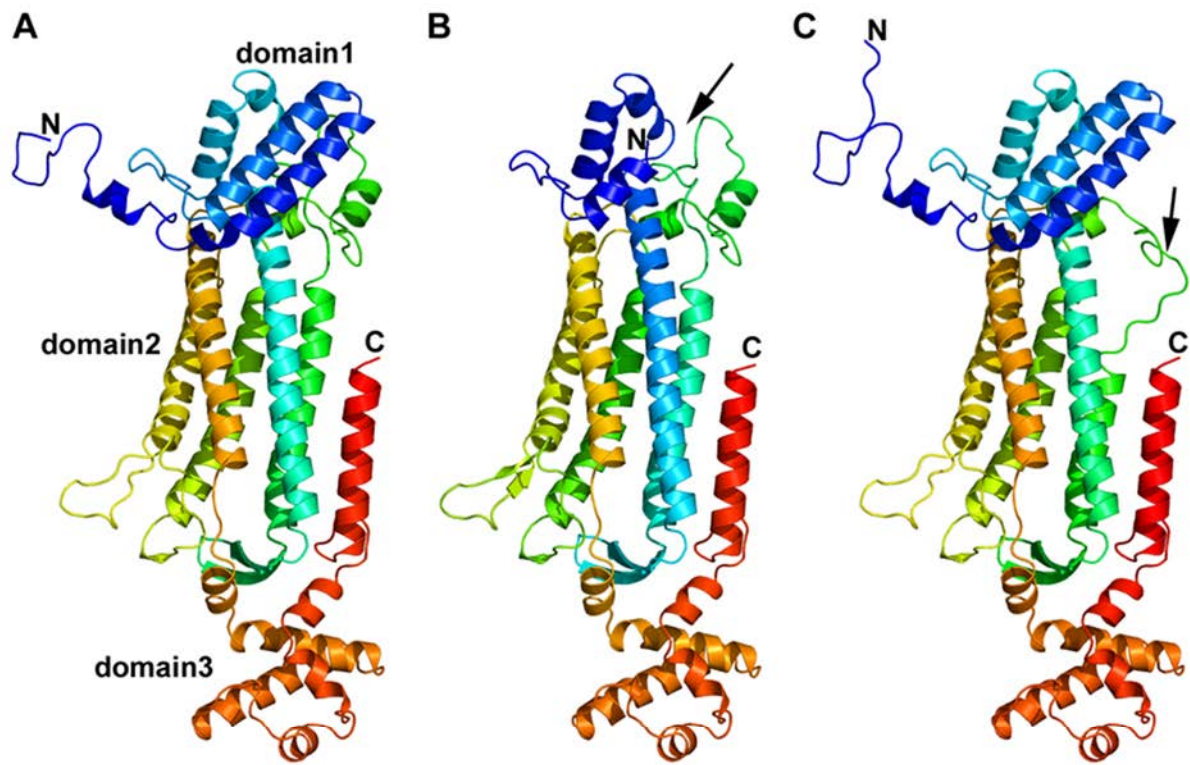


Figure 6

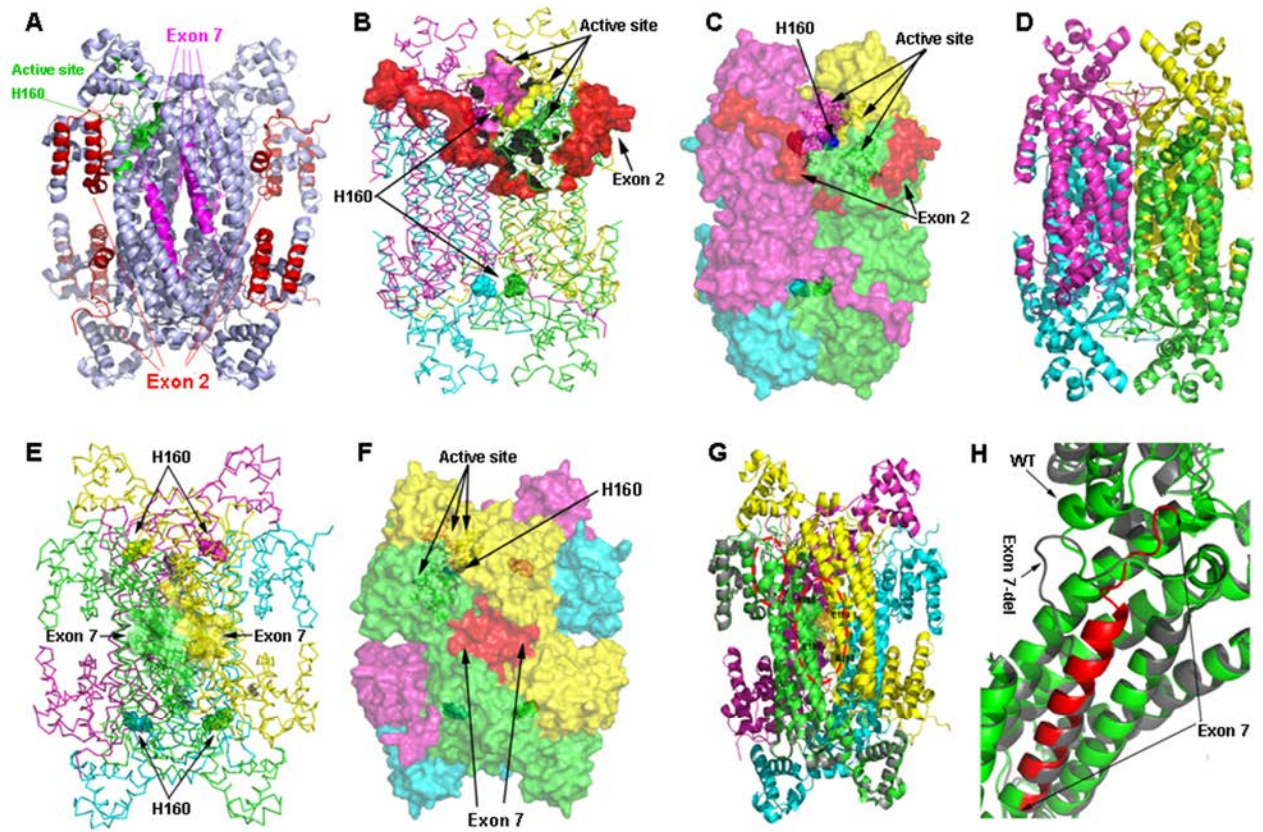


Figure 7

